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Article

Cowpox Virus Exploits the Endoplasmic Reticulum Retention Pathway to Inhibit MHC Class I Transport to the Cell Surface

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SUMMARY

Major histocompatibility complex (MHC) class I molecules assemble with peptides in the ER lumen and are transported via Golgi to the plasma membrane for recognition by T cells. Inhibiting MHC assembly, transport, and surface expression are common viral strategies of evading immune recognition. Cowpox virus, a clinically relevant orthopoxvirus, downregulates MHC class I expression on infected cells. However, the viral protein(s) and mechanisms responsible are unknown. We identify CPXV203 as a cowpox virus protein that associates with fully assembled MHC class I molecules and blocks their transport through the Golgi. A C-terminal KTEL motif in CPXV203 closely resembles the canonical ER retention motif KDEL and is required for CPXV203 function, indicating that a physiologic pathway is exploited to retain MHC class I in the ER. This viral mechanism for MHC class I downregulation may explain virulence differences between clinical isolates of orthopoxviruses.

INTRODUCTION

Orthopoxviruses are clinically important infectious agents. Variola virus (VARV) causes smallpox, one of the most devastating human diseases. Naturally occurring smallpox was eradicated in the late 20th century by a worldwide vaccination program using vaccinia virus (VACV) (Fenner et al., 1988). However, the potential threat of deliberate reintroduction of VARV still exists (Smith and McFadden, 2002). Other orthopoxviruses, such as cowpox virus (CPXV) and monkeypox virus (MPXV) cause human disease through zoonosis (Lewis-Jones, 2004). CPXV is endemic in Europe, where occasional transmission to humans often occurs via contact with infected domestic cats (Chantrey et al., 1999; Lewis-Jones, 2004). MPXV causes a smallpox-like disease in humans (Breman et al., 1980; Jezek et al., 1987). Since the first case report in 1970, sporadic outbreaks of human monkeypox have occurred in Central and West Africa and recently in the United States, with Central African clades demonstrating more virulence (Breman et al., 1980; Foster et al., 1972; Ladnyj et al., 1972; Reed et al., 2004). The basis for difference in virulence between MPXV clades is largely unknown and under active investigation (Chen et al., 2005; Likos et al., 2005). However, it is likely that viral immune evasion strategies contribute to differences in virulence.

In their large DNA genomes, poxviruses encode some of the most extensive inventories of genes dedicated to evading the host immune system. Targets of these genes are comprehensive, including primary mediators of both innate and adaptive immunity such as complement system, interferons, inflammatory cytokines, and chemokines (Seet et al., 2003). Among the orthopoxviruses, CPXV encodes the most complete repertoire of immunomodulatory genes, and comparative analysis of orthopoxvirus genomes suggested that CPXV or CPXV-like virus was the ancestor of all the modern orthopoxviruses (Meyer et al., 2002; Shchelkunov et al., 2002). Therefore, CPXV is ideal to study orthopoxvirus-encoded immune evasion mechanisms.

The important role of cytotoxic T lymphocytes (CTL) in controlling virus infection is highlighted by viral evasion strategies. CTLs usually recognize antigens processed by the major histocompatibility complex (MHC) class I antigen presentation pathway (Townsend et al., 1989). MHC class I molecules, composed of heavy chain and β2-microglobulin (β2m), fold and assemble in the lumen of the endoplasmic reticulum (ER) with the help of chaperones (Pamer and Cresswell, 1998). Peptides generated by the proteasomes in the cytosol are translocated into the ER lumen by the transporter associated with antigen processing (TAP). Upon peptide loading with the aid of the loading complex including TAP, tapasin, ERp57, and calreticulin, MHC class I molecules leave the ER and travel to the plasma membrane through the secretory pathway. On the cell surface, they present peptides sampled from the endogenous protein repertoire including virus-derived peptides. Upon recognition of viral peptide-MHC class I complex through the T cell receptor, virus-specific CTLs kill the infected cells and produce inflammatory cytokines such as interferon-γ and tumor necrosis factor-α to prevent further viral spread (Guidotti and Chisari, 1996). To

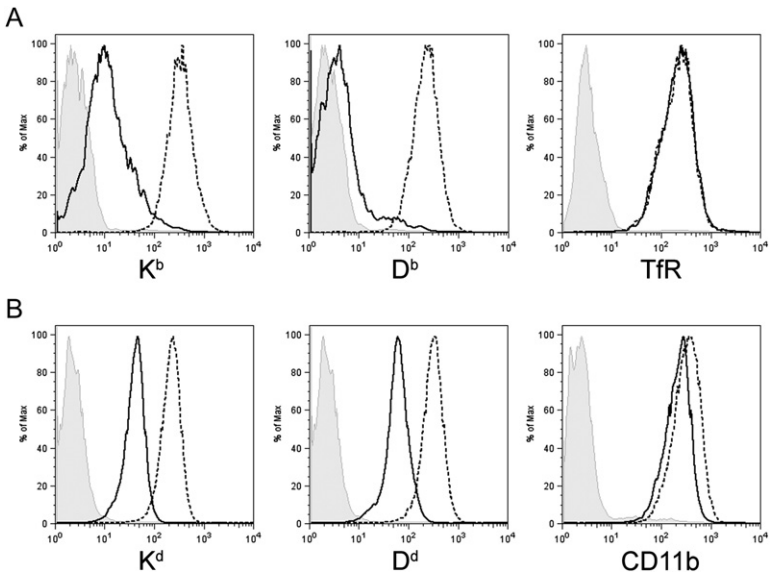

Figure 1. Selective Downregulation of MHC Class I Molecules on CPXV-Infected Cells

MC57G fibroblast cells (A) and macrophages derived from BALB/c bone marrow (B) were either mock infected (dashed line) or infected with CPXV at a multiplicity of infection (MOI) of 5 (solid line). At 24 hr postinfection, MC57G cells were stained with antibodies to K^b, D^b, and transferrin receptor (TfR), and macrophages were stained with antibodies to K^d, D^d, and CD11b. Stained cells were analyzed by flow cytometry. Gray-filled histograms represent isotype-control antibody staining.

avoid the antiviral action of CTLs, many viruses, notably the large DNA viruses, have evolved evasion mechanisms targeting the MHC class I antigen presentation pathway (Tortorella et al., 2000).

While the downregulation of MHC class I molecules on the cell surface protects virus-infected cells from recognition and killing by CD8 T cells, it leaves the infected cells susceptible to lysis by natural killer (NK) cells owing to the reduced engagement of MHC class I molecules by NK cell inhibitory receptors. The recent discovery of orthopoxvirus MHC class I-like protein (OMCP) that acts as a competitive antagonist of an NK cell activating receptor NKG2D (Campbell et al., 2007) explains how orthopoxviruses can counteract NK cell attack and predicts that orthopoxviruses may downregulate MHC class I. However, until recently, there was no report that orthopoxviruses specifically modulate MHC class I expression.

Recently, CPXV was reported to evade recognition by CPXV-specific $CD8^+$ T cells (Dasgupta et al., 2007). In this study, the authors demonstrated that CPXV induces downregulation of human MHC class I molecules from the cell surface by inhibiting the intracellular transport of peptide-loaded MHC class I (Dasgupta et al., 2007). However, the molecular basis of this evasion mechanism is largely unknown because the CPXV protein responsible for the modulation of MHC class I expression was not identified.

Here we used a comparative genomics-based approach and a gain-of-function screening to identify CPXV gene(s) capable of MHC class I downregulation. Interestingly, we found that CPXV encodes more than one gene that can modulate MHC class I expression. We report the identification and characterization of CPXV203 protein, which is sufficient for the downregulation of mouse and human MHC class I molecules. Mechanistically, CPXV203 uses the physiologic KDEL pathway to specifically retain MHC class I molecules in the ER.

RESULTS

Downregulation of MHC Class I on the Surface of CPXV-Infected Cells

Despite its name, the natural reservoir hosts of CPXV are wild rodents (Chantrey et al., 1999). To study CPXV-mediated MHC class I downregulation in a relevant murine host system, we infected various murine cell types with CPXV. MC57G fibroblast cells infected with CPXV strain Brighton Red (BR) displayed dramatically reduced surface expression of both K^b and D^b at 24 hr postinfection compared to the mock-infected cells (Figure 1A). We also observed downregulation of K^b and D^b on C57BL/6-derived bone marrow-derived macrophages (BMMs) and dendritic cells infected with CPXV (Figure S1A in the Supplemental Data available with this article online). Furthermore, the expression levels of K^d and D^d on the BALB/c-derived BMMs (Figure 1B) and K^k and D^k in L929 fibroblast cells (Figure S1A) were also reduced upon CPXV infection. Interestingly, cell surface expression of transferrin receptor (Figure 1A), CD11b (Figure 1B), CD11c, and CD44 (Figure S1A) remained unaffected at 24 hr postinfection, suggesting that downregulation was specific to MHC class I molecules. In summary, CPXV induces downregulation of broad range of murine MHC class I molecules from various cell types.

We also examined the time course of MHC class I downregulation in MC57G cells. Similar to what was reported for human MHC class I molecules (Dasgupta et al., 2007), the decrease of surface K^b expression was detectable at 4 hr postinfection and progressed further until 24 hr postinfection (data not shown). Furthermore, blocking viral intermediate and late gene transcription by AraC treatment did not abolish MHC class I downregulation (Figure S1B). Together, these data indicated that an early gene product(s) of CPXV is responsible for the phenotype.

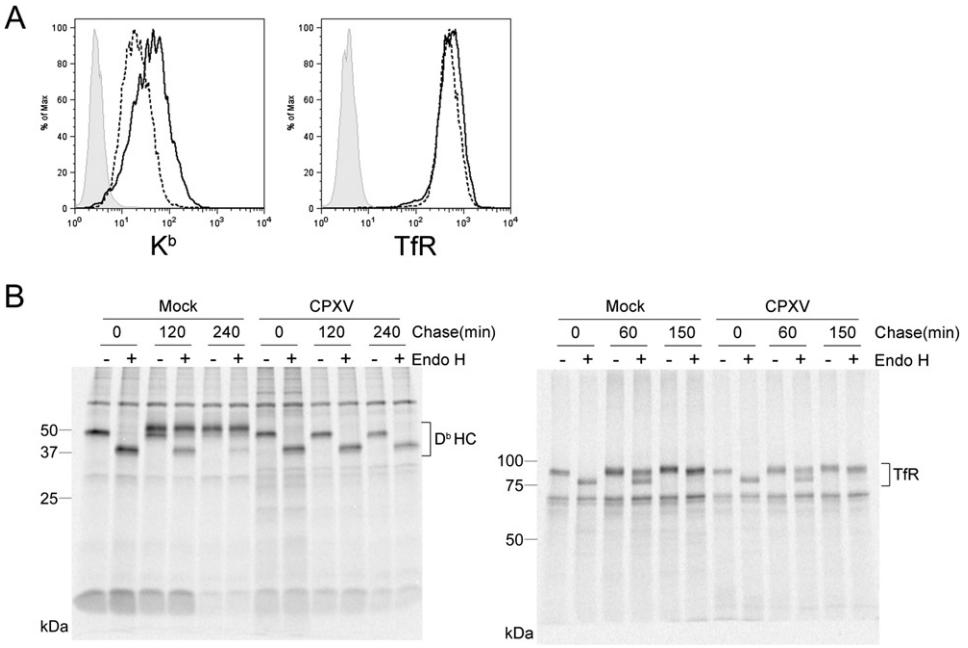

Figure 2. Impaired Intracellular Transport of MHC Class I in CPXV-Infected Cells

(A) MC57G cells were either mock infected (dashed line) or infected with CPXV at a MOI of 5 (solid line). At 24 hr postinfection, cells were fixed, permeabilized, and stained with antibodies to K^b or transferrin receptor (TfR). Stained cells were analyzed by flow cytometry. Gray-filled histograms represent isotype-control antibody staining.

(B) MC57G cells were either mock infected or infected with CPXV at a MOI of 5. At 6 hr postinfection, cells were pulse labeled for 10 min with [^{35}S]cysteine/methionine and chased for the indicated time periods. D^b heavy chain (HC) and transferrin receptor (TfR) were immunoprecipitated from the cell lysates and either Endo H-treated or mock treated prior to SDS-PAGE. A representative result of three independent experiments is shown.

Impaired Intracellular Trafficking of MHC Class I Molecules in CPXV-Infected Cells

To elucidate the mechanism by which CPXV interferes with MHC class I expression, we examined whether the decrease of surface expression simply reflected a reduction of total cellular MHC class I level. Surprisingly, in contrast to the severely reduced surface expression, we detected increased amounts of K^b in CPXV-infected MC57G cells than in the mock-infected cells by intracellular staining (Figure 2A). The total level of transferrin receptor remained unchanged, indicating that the increase of total K^b expression was not due to a cytopathic effect (Figure 2A). These data argued against an accelerated degradation of MHC class I molecules as the mechanism for downregulation of their surface level.

We then investigated a potential defect in the intracellular transport of MHC class I molecules by examining Endoglycosidase H (Endo H) sensitivity of newly synthesized MHC class I heavy chains. In the mock-infected MC57G cells, most D^b heavy chains acquired Endo H resistance within the 240 min chase period, consistent with glycosylation during transit through the Golgi complex. However, during the same time period, D^b heavy chains in CPXV-infected cells remained Endo H sensitive, indicating a block in the trafficking to the medial Golgi (Figure 2B). Similarly, newly synthesized K^b heavy chains remained Endo H sensitive in CPXV-infected cells (data not shown). These results were not due to a general impairment of protein trafficking because trafficking of transferrin receptors remained unaffected in CPXV-infected cells (Figure 2B). Taken together, these data indicated that CPXV induces selective retention of MHC class I molecules in the pre-Golgi compartments, causing MHC class I downregulation at the cell surface.

Identification of *CPXV203* as a CPXV-Encoded MHC Class I Modulator

To further investigate the molecular basis for this potential immune evasion mechanism, we searched for a CPXV gene product(s) capable of modulating MHC class I expression. Preliminary experiments demonstrated that other closely related orthopoxviruses, VACV strain Western Reserve (WR) and ectromelia virus (ECTV) strain Moscow, do not induce selective MHC class I downregulation (data not shown), indicating that only CPXV, but not VACV or ECTV, encodes protein(s) mediating this potential immune evasion. We used the Poxvirus Bioinformatics Resource Center (PBRC) database to compare the genome of CPXV BR to those of VACV WR and ECTV Moscow. Candidate open reading frames (ORFs) were selected if (1) they are present in CPXV but not in the other viruses, or (2) they encode proteins with less than 80% amino acid identity to the VACV or ECTV orthologs. These candidate genes were cloned from genomic DNA and expressed individually in BaF/3 cells using a retroviral expression system.

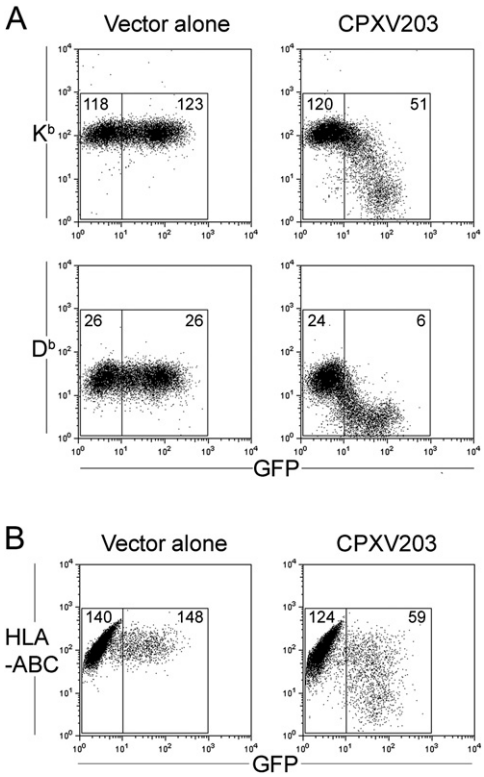

Figure 3. Downregulation of Murine and Human MHC Class I by CPXV203

C1498 cells (A) and Jurkat cells (B) that were transduced with either control retroviral vector alone or *CPXV203* were stained with antibodies to K^b, D^b, or HLA-ABC and analyzed by flow cytometry. The retroviral vectors used for transduction encode GFP, and thus only successfully transduced cells are GFP positive. The numbers in the gates indicate mean fluorescence intensity of MHC class I molecules for GFP-negative and -positive populations.

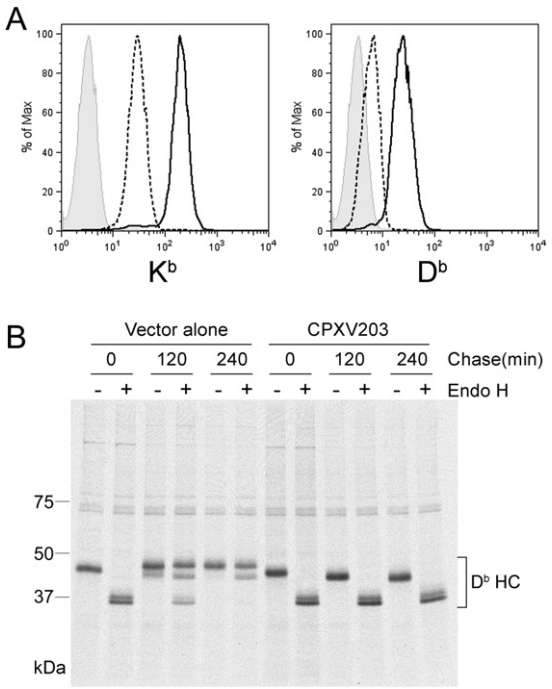

Figure 4. Inhibition of MHC Class I Trafficking by CPXV203

(A) C1498 cells transduced with either vector alone (dashed line) or *CPXV203* (solid line) were subjected to intracellular staining with antibodies to K^b and D^b. Gray-filled histograms represent isotype-control antibody staining.

(B) The same set of cells in (A) was metabolically labeled for 10 min and chased for the indicated periods of time. D^b was immunoprecipitated from the cell lysates and treated with Endo H where indicated. Precipitated proteins were separated by SDS-PAGE and visualized by autoradiography. A representative result of three independent experiments is shown.

Among the 21 genes tested, the expression of *CPXV203* induced downregulation of K^d from the surface of BaF/3 cells (Figure S2A). To confirm this result, *CPXV203* was expressed in another murine cell line, C1498, using a bicistronic retroviral expression vector encoding enhanced green fluorescent protein (GFP) for ready detection of transduced cells. C1498 cells transduced with *CPXV203* had dramatically reduced levels of K^b and D^b on the cell surface in comparison with the nontransduced cells or cells transduced with the vector alone (Figure 3A). The expression levels of other surface proteins were unaffected by *CPXV203* transduction (data not shown). Furthermore, transduction of *CPXV203* in the human cell line Jurkat induced downregulation of human MHC class I molecules on the cell surface (Figure 3B). Similar results were obtained using another human cell line 293T (data not shown). Thus, the gene product of *CPXV203* can modulate both murine and human MHC class I expression.

To understand the mechanism by which CPXV203 modulates surface MHC class I expression, we examined the total amount of MHC class I in *CPXV203*-transduced cells by intracellular staining. As seen with CPXV-infected cells, total levels of K^b and D^b were increased in *CPXV203*-transduced cells compared to the cells transduced with vector alone (Figure 4A). Moreover, trafficking of K^b (Figure S2B) and D^b (Figure 4B) through the Golgi complex was impaired in *CPXV203*-transduced cells. Therefore, CPXV203 induces accumulation and retention of MHC class I molecules in the pre-Golgi compartments, conferring the phenotype of CPXV-infected cells.

ER Localization of CPXV203 and the Physical Association with MHC Class I

To understand how CPXV203 achieves intracellular retention of MHC class I, we examined the subcellular localization of CPXV203 protein by immunofluorescence microscopy. CPXV203 with the insertion of HA epitope between the residue G107 and A108 (CPXV203-HA) was still capable of downregulating MHC class I molecules (data not shown). Staining of CPXV203-HA in B6/WT-3 cells with HA-specific antibody revealed a reticular pattern colocalized with that of calnexin, an ER-resident protein (Figure 5A). Furthermore, CPXV203-HA did not colocalize with the Golgi marker giantin (Figure 5A). These data indicated that CPXV203 protein resides in the ER.

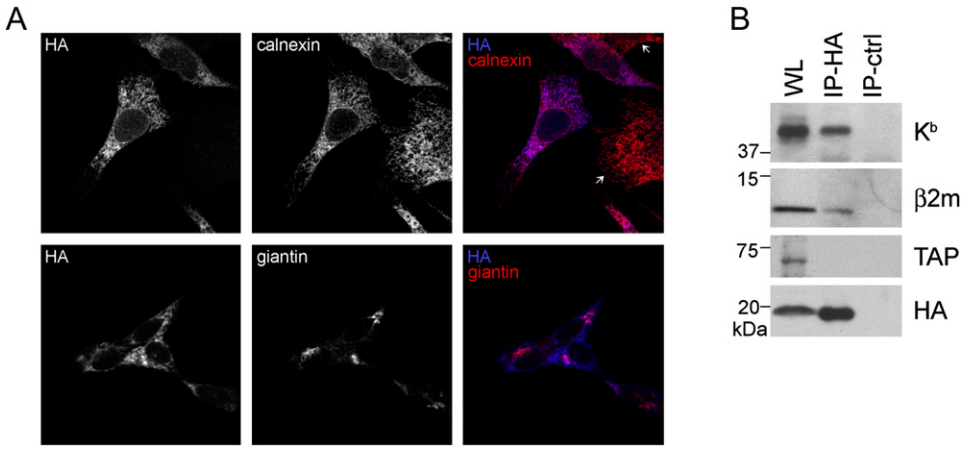

Figure 5. Physical Association between CPXV203 and MHC Class I Molecules

(A) B6/WT-3 cells were transduced with CPXV203-HA and stained with antibodies to HA, an ER marker calnexin, or a Golgi marker giantin. Nontransduced cells that express calnexin but not CPXV203-HA are indicated by arrows.

(B) C1498 cells transduced with CPXV203-HA were lysed with 1% digitonin. Whole lysate (WL) and immunoprecipitates of anti-HA antibody (IP-HA) or isotype-control antibody (IP-ctrl) were separated by SDS-PAGE and examined by western blot analysis for the presence of K^b, β2m, TAP, and HA. A representative result of more than three independent experiments is shown.

Based on the ER localization of CPXV203, we hypothesized two potential mechanisms by which CPXV203 retains MHC class I molecules in the ER. First, CPXV203 might interfere with the peptide loading of MHC class I molecules, thereby their egress from the ER. However, incubation with saturating concentration of K^b-binding peptide VNVDYSKL (Sasada et al., 2001) did not restore the reduced surface K^b expression on *CPXV203*-transduced cells (Figure S3). Furthermore, we observed increased amounts of peptide-loaded K^b and D^b in *CPXV203*-transduced C1498 cells, as detected by immunoprecipitation with conformation-specific antibodies (B8-24-3 for peptide-associated K^b and B22-249 for peptide-associated D^b) (data not shown). These data suggested that a defect in peptide loading is unlikely to account for the ER retention of MHC class I by CPXV203.

The accumulation of peptide-loaded MHC class I molecules in the presence of CPXV203 suggested, alternatively, that CPXV203 might physically associate with peptide-loaded MHC class I molecules, thereby holding them in the ER. To test this hypothesis, we performed coimmunoprecipitation experiments in C1498 cells expressing CPXV203-HA. We found K^b heavy chain and β2m (Figure 5B) coprecipitated with the anti-HA antibody but not with the isotype-control antibody. TAP, one of the components of the peptide-loading complex, was not detected in the anti-HA precipitates (Figure 5B). Taken together, these data indicated that CPXV203 physically associates with fully assembled MHC class I molecules, after the latter dissociate from the peptide-loading complex upon peptide acquisition.

Essential Role of KTEL in the Localization and Function of CPXV203

The deduced CPXV203 protein contains a putative N-terminal signal peptide and no predicted transmembrane domain. The final four C-terminal residues (KTEL) closely resemble the canonical ER retention motif KDEL. In animal cells, soluble proteins bearing KDEL motif at their C termini are constantly retrieved upon escape from the ER by the KDEL receptor, which recycles between the *cis*-Golgi and the ER (Pelham, 1996). Variants of the KDEL motif, including KTEL, with a similar function have been described (Andres et al., 1990, 1991; Vennema et al., 1992). Thus, the presence of KTEL sequence at the C terminus of CPXV203 was consistent with the ER localization of the protein.

We hypothesized that, if CPXV203 retains MHC class I in the ER via the KTEL motif, deletion of this motif from CPXV203 would lead to the release of the CPXV203/MHC class I complex from the ER. Indeed, KTEL-deleted CPXV203 did not downregulate MHC class I molecules (Figure 6A). To test whether the functional defect of KTEL-deleted CPXV203 was due to an inefficient ER retention of CPXV203, we examined the fate of newly synthesized full-length or KTEL-deleted CPXV203-HA by metabolic labeling. As we predicted, KTEL-deleted CPXV203-HA was rapidly secreted into the supernatant, while the full-length form remained mostly intracellular (Figures 6B and 6C). In conclusion, the KTEL motif of CPXV203 is essential for the functional effect of CPXV203 on MHC class I.

Partial Rescue of MHC Class I Downregulation by the Deletion of CPXV203

To study the role of CPXV203 in CPXV-mediated MHC class I downregulation, we generated a recombinant virus lacking *CPXV203* (CPXVΔ203) by replacing the *CPXV203* coding region with VACV I1L promoter-driven GFP, which was flanked by two loxP sites for ready deletion of the GFP cassette (Figure 7A). Deletion of *CPXV203* was confirmed by PCR, sequencing, and immunoblotting (Figure 7B).

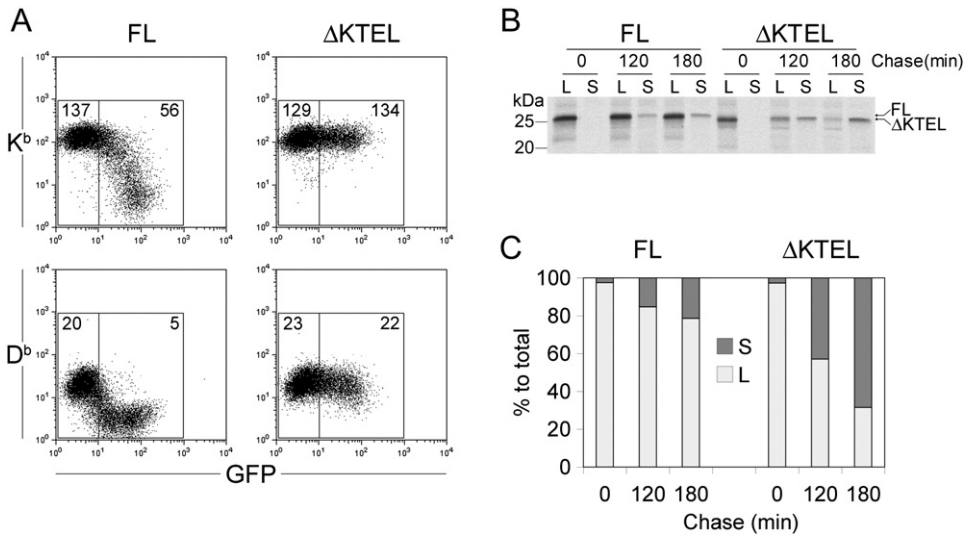

Figure 6. Crucial Role of C-Terminal KTEL Motif in the Function of CPXV203

(A) Surface expression levels of K^b and D^b on C1498 cells transduced with either full-length (FL) or KTEL-deleted (ΔKTEL) CPXV203 were analyzed by flow cytometry. The numbers in the gates indicate mean fluorescence intensity of MHC class I molecules for GFP-negative and -positive populations.

(B) C1498 cells were transduced with either full-length CPXV203-HA (FL) or KTEL-deleted CPXV203-HA (ΔKTEL). Cells were metabolically labeled for 10 min and chased for the indicated time periods. CPXV203 was immunoprecipitated from either the cell lysates (L) or the culture supernatants (S) with anti-HA antibody.

(C) Band intensities of L and S fractions of the gel in (B) were quantified and plotted as percentage of total protein level (L+S) for each time point.

CPXVΔ203 replicated normally in Vero cells as compared to the wild-type CPXV (CPXV-WT) (data not shown).

MC57G cells infected with CPXVΔ203 demonstrated less reduction of surface K^b than those infected with CPXV-WT (Figure 7C). This was not due to a difference in multiplicity of infection because both viruses infected MC57G cells similarly, as measured by poxvirus hemagglutinin staining (Figure 7C). Deletion of the GFP cassette from CPXVΔ203 by Cre recombinase did not affect the phenotype of CPXVΔ203 (data not shown). These data confirmed that CPXV203 plays a major, nonredundant role in MHC class I downregulation by CPXV.

Interestingly, CPXVΔ203 still induced substantial downregulation of K^b on the cell surface (Figure 7C). Moreover, K^b heavy chains in CPXVΔ203-infected cells were mostly Endo H sensitive as in CPXV-WT-infected cells (Figure 7D). These data indicated that, in addition to *CPXV203*, CPXV encodes another gene(s) that can modulate MHC class I expression by blocking intracellular transport.

DISCUSSION

Here, we characterized the downregulation of murine MHC class I molecules by CPXV. The broad effect of CPXV on the MHC class I levels of various cell types elevates the importance of understanding this immune evasion mechanism, since CPXV is known to infect diverse cell types including epithelial cells, fibroblasts, and macrophages in vivo (Martinez et al., 2000). Furthermore, we demonstrated that murine MHC class I downregulation by CPXV shares similarities with the human system (Dasgupta et al., 2007). In both murine and human cells infected with CPXV, downregulation of MHC class I occurs with similar kinetics and is independent of the intermediate and late gene expression. The assembly of MHC class I molecules appears to be normal in both CPXV-infected murine and human cells, although we observed increased total levels of K^b, which was not reported for the human MHC class I molecules. It is not clear whether it is due to a difference in species, cell lines, or multiplicity of infection. Importantly, we found that CPXV inhibits the intracellular trafficking of murine MHC class I molecules, as it does to the human MHC class I molecules (Dasgupta et al., 2007). These data strongly suggest that CPXV uses a same set of genes and mechanisms to modulate murine and human MHC class I expression.

Our combined findings reported here demonstrate the mechanism by which CPXV203 modulates MHC class I expression. CPXV203 protein associates with MHC class I heavy chain and β2m, but not with TAP, suggesting that this association likely occurs after fully assembled MHC class I molecules disassociate from TAP and other members of the peptide-loading complex. It is also consistent with the intracellular accumulation of peptide-loaded MHC class I molecules. Given that CPXV203 is a lumenal protein, it must be interacting with the ectodomain of MHC class I, although this interaction may not be direct, i.e., it may involve other host proteins. Once associated, the CPXV203/MHC class I complex is ER retained by the KTEL sequence of CPXV203 presumably interacting with KDEL receptor.

The specificity of CPXV203-mediated immune evasion mechanism is particularly intriguing. The expression of CPXV203 does not appear to have a "dominant-negative"

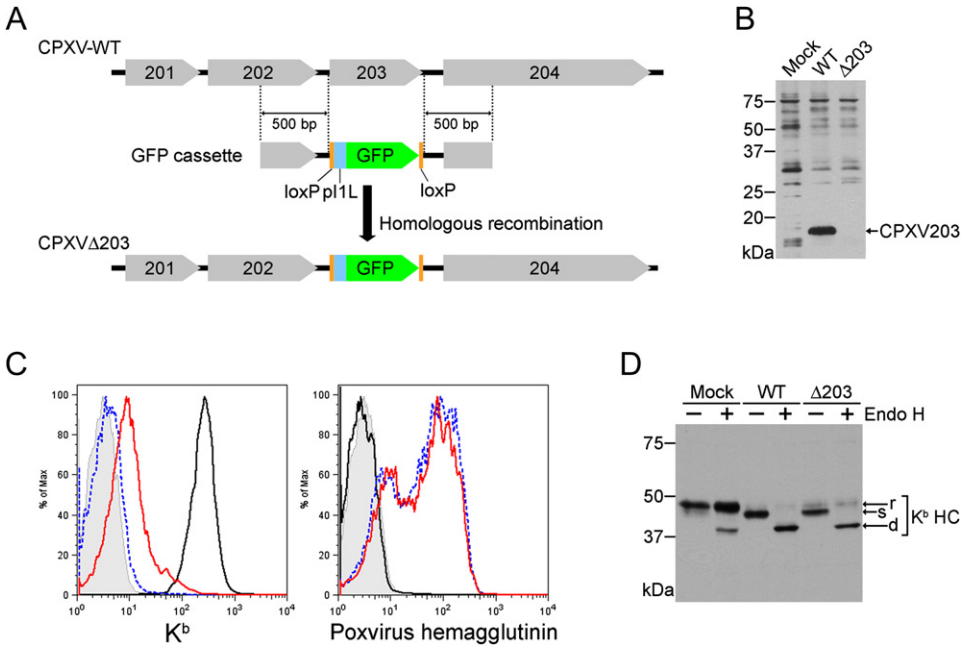

Figure 7. Partial Rescue of MHC Class I Downregulation by the Deletion of CPXV203

(A) CPXVΔ203 was generated by homologous recombination between CPXV-WT and the GFP cassette flanked by two 500 bp homologous arms. The GFP cassette is composed of VACV I1L promoter (pI1L) followed by GFP coding sequence and two loxP sites. Gray-filled arrows represent coding regions of indicated ORFs, and the direction of arrows indicates the direction of transcription.

(B) MC57G cells were mock infected (Mock) or infected with CPXV-WT (WT) or CPXVΔ203 (Δ203) at a MOI of 5. At 24 hr postinfection, cells were lysed and the lysates were analyzed by immunoblotting using polyclonal antibody raised against a C-terminal peptide of CPXV203.

(C) MC57G cells were either mock infected (black) or infected with CPXV-WT (dashed blue) or CPXVΔ203 (solid red). At 24 hr postinfection, cells were stained with antibodies to K^b or poxviral hemagglutinin. Gray-filled histograms represent isotype-control staining.

(D) Cell lysates were prepared from MC57G cells that were mock infected (Mock) or infected with CPXV-WT (WT) or CPXVΔ203 (Δ203) at a MOI of 5 for 24 hr, and treated with Endo H where indicated. Western blot analysis was performed using an anti-K^b antibody. Protein bands corresponding to the Endo H-resistant (r), -sensitive (s), or -digested (d) K^b heavy chain (HC) are indicated.

effect by saturating the KDEL receptor and thus aborting the molecular chaperones retained in the ER by this mechanism. Calreticulin has a KDEL motif and ERp57 has a QEDL, and both of them are implicated in facilitating MHC class I assembly in the ER. However, we did not find a significant difference in the expression levels of these chaperones in the presence or absence of CPXV203 (data not shown), and the assembly of MHC class I appears to be normal in *CPXV203*-transduced cells. Perhaps the high cargo retrieval capacity of the KDEL receptor (Pelham, 1996), and the lower efficiency of KTEL as a retention signal compared to KDEL (Vennema et al., 1992), can explain how CPXV203 retains MHC class I molecules without affecting the retention of the ER chaperones containing KDEL-like motifs. After all, a complete block of this retention mechanism may not be in the virus' best interest because KDEL-bearing ER chaperones may facilitate the folding of viral proteins. Taken together, our data strongly suggest that CPXV203 exploits a physiological pathway of ER retention without disrupting overall ER quality control.

CPXV203 is highly conserved among some of the orthopoxviruses, including other strains of CPXV, MPXV, camelpox virus, and taterapox virus, but its orthologs are truncated or absent in other orthopoxviruses (Figure S4). This may be not surprising because there is no single common immunomodulatory gene that can be found in all poxviruses (Seet et al., 2003). However, it is noteworthy that OMCP, the recently discovered antagonist of NK cells, is conserved only among CPXV and MPXV that also encode CPXV203 or its ortholog (Campbell et al., 2007). Considering that OMCP may be capable of compensating for NK cell activation caused by CPXV203-mediated MHC class I downregulation, it is attractive to speculate that these immunomodulatory genes might have coevolved to complement each other.

We speculate that our study may help understand a virulence difference between MPXV strains. Human monkeypox cases as well as infection studies of cynomolgus monkeys and ground squirrels demonstrated that West African MPXV isolates were less virulent than Congo basin isolates (Breman et al., 1980; Chen et al., 2005; Foster et al., 1972; Ladnyj et al., 1972; Sbrana et al., 2007). Through genome comparison of several West African and Congo basin MPXV isolates, five genomic regions were identified that may explain the observed differences in pathogenicity between these two clades (Chen et al., 2005; Likos et al., 2005). Proteins encoded in these regions include orthologs of previously demonstrated poxvirus immunomodulatory proteins such as the complement control protein and interleukin-1β receptor

homolog. Interestingly, one of these candidate regions contains the ortholog of *CPXV203*. Congo basin isolates encode a full-length ortholog with the conserved C-terminal KTEL motif, whereas West African isolates encode a truncated form, containing only the N-terminal one-third of the full-length protein. Our preliminary experiments suggest that the full-length ortholog *B10R* of Congo basin strain Zaire, but not the truncated form of West African strain COP-58, can downregulate human MHC class I molecules from the cell surface (data not shown). It is tempting to speculate that the absence of a functional *CPXV203* ortholog, and thus the inefficient CTL evasion, might contribute to the attenuated phenotype of West African isolates compared to that of Congo basin isolates.

CPXV203 is also distantly related to M-T4 protein of myxoma virus, a leporipoxvirus causing rabbit myxomatosis (Figure S4). M-T4 contains KDEL-like retention motif RDEL and was shown to localize in the ER (Barry et al., 1997). The phenotype of M-T4-deleted virus suggested that M-T4 is a virulence factor and prevents apoptosis in infected lymphocytes, but the C-terminal RDEL motif was not required for these functions (Hnatiuk et al., 1999). Interestingly, myxoma virus was shown to downregulate MHC class I on the cell surface by targeting MHC class I molecules for degradation in late endosomes and/or lysosomes (Boshkov et al., 1992; Zuniga et al., 1999), and myxoma virus leukemia-associated protein (MV-LAP) was demonstrated to be responsible for MHC class I endocytosis and degradation in myxoma virus-infected cells (Guerin et al., 2002). Considering the similarity between CPXV203 and M-T4, such as the presence of the KDEL-like motif and multiple conserved cysteines, it would be interesting to test whether M-T4 also plays a role in myxoma virus-mediated MHC class I downregulation. Vice versa, whether CPXV203 has an M-T4 like antiapoptotic function needs to be determined.

Even in the absence of CPXV203, CPXV blocks the trafficking of most MHC class I molecules through the Golgi complex, indicating it encodes as yet another gene(s) that can retain MHC class I molecules in the pre-Golgi compartments. Perhaps this is not surprising, since other large DNA viruses contain multiple ORFs that target different steps of MHC class I biosynthesis pathway. The MHC class I retention mechanism(s) employed by this gene(s) must be different from that of CPXV203 because *CPXV203* is the only CPXV BR gene containing a C-terminal KDEL-like sequence. We are currently investigating the identity of this CPXV gene(s). Although in vivo validation of the role that CPXV-induced MHC class I downregulation plays in CTL evasion and in virulence will have to await the identification and deletion of all individual genes that are involved, our work provides a stepping stone for understanding CPXV pathogenesis.

EXPERIMENTAL PROCEDURES

Cell Lines and Virus

Cell lines MC57G, L929, C1498, Jurkat, 293T, Vero, and CV-1 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in recommended media. Bone marrow-derived macrophages were generated by culturing single-cell suspension of bone marrow from C57BL/6 or BALB/c mice in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum and L929 cell supernatant. B6/WT-3 (Lybarger et al., 2003) is an SV-40 transformed C57BL/6 murine embryo fibroblast line. Cowpox virus strain Brighton Red was obtained from ATCC and amplified in Vero cells. Viruses were purified from the infected Vero cell lysates by centrifugation through 36% sucrose solution. Titers of virus stocks were quantified by plaque assays using CV-1 cells.

Flow Cytometry

The following monoclonal antibodies were used for flow cytometry; AF6-88.5 for K^b, KH95 for D^b, SF1-1.1 for K^d, 34-2-12 for D^d, C2 for transferrin receptor, M1/70 for CD11b (all from BD Biosciences), W6/32 (ATCC) for HLA-ABC, and IH831 (a gift of G. Karupiah, Australian National University) for poxvirus hemagglutinin. For intracellular staining, cells were fixed and permeabilized with 4% (w/v) paraformaldehyde/0.1% (w/v) saponin prior to antibody staining. All flow cytometric analyses were performed using a FACSCalibur (Becton Dickinson), and the data were analyzed using FlowJo software (Tree Star).

Metabolic Labeling and Immunoprecipitation

Cells subject to metabolic labeling were incubated with PRO-MIX L-[^{35}S] in vitro cell-labeling mix (Amersham Biosciences) at 500 μCi/ml for 10 min. After the labeling medium was removed, cells were incubated in the chase medium with excess unlabeled methionine/cysteine (5 mM each) for the indicated chase periods. Cells were washed, and were lysed in 0.5% NP-40 (Sigma) in Tris-buffered saline (TBS) (10 mM Tris [pH 7.6], 150 mM sodium chloride) with protease inhibitor cocktail (Roche). Postnuclear lysates were precleared and incubated for 2 hr at 4°C with protein A sepharose (PAS) (RepliGen) or protein G sepharose (PGS) (Amersham Biosciences) that were preincubated with the following mAbs: anti-D^b 28-14-8 (ATCC), anti-K^b B8-24-3 (ATCC), anti-transferrin receptor YTA74.4 (Serotec), or anti-HA 16B12 (Covance). After stringent washing, precipitated proteins were eluted from PAS or PGS and subjected to SDS-PAGE. For the trafficking studies, eluted proteins were treated with 10 mU of Endoglycosidase H (Roche) for 2 hr at 37°C prior to SDS-PAGE. Protein band intensities were quantified by ImageQuant TL (Amersham Biosciences).

Cloning of CPXV ORFs and Retroviral Transduction

Genomes of CPXV strain BR, VACV strain WR, and ECTV strain Moscow were compared using Ortholog Comparisons function of the PBRC website (http://www.poxvirus.org/). For the transduction of murine cell lines, candidate ORFs including *CPXV203* were cloned from CPXV genomic DNA into a bicistronic retroviral expression vector pMXsIG (T. Kitamura, University of Tokyo), which contains an internal ribosomal entry site followed by a second cistron for GFP. Plat-E cells (T. Kitamura, University of Tokyo) were transfected with individual construct using FuGENE6 (Roche). The supernatants containing retroviruses were harvested at day 2 and used to transduce the target cells. For the transduction of human cell lines, *CPXV203* was subcloned into pHSPG vector (L. Su, University of North Carolina), which contains pGK-driven GFP, and amphotropic phoenix cells (ATCC) were used to produce retroviruses. Hexadimethrine bromide (Sigma) was also added to the final concentration of 5–10 μg/ml in the viral supernatants to facilitate infection.

Confocal Laser Scanning Microscopy

B6/WT-3 cells grown on glass coverslips were fixed with 2% (w/v) paraformaldehyde and permeablized with 0.2% (w/v) saponin. To block nonspecific binding of antibodies, all staining was performed in the presence of 10% (v/v) goat serum. Cells were stained with mouse anti-HA 16B12, rabbit anti-calnexin (Sigma), or rabbit anti-giantin, followed by Alexa Fluor 633-conjugated goat anti-mouse IgG and Alexa Fluor 568-conjugated goat anti-rabbit IgG (Molecular

Probes). Stained cells were analyzed with laser scanning confocal microscope LSM510 (Zeiss).

Coimmunoprecipitation

Cells were lysed in TBS buffer containing 1% digitonin (Wako) and 20 mM iodoacetamide (Sigma). After preclearing over PGS, postnuclear lysates were incubated with anti-HA 16B12 or mouse IgG1 isotype-control 9E10 (ATCC) bound PGS for 2 hr at 4°C. Proteins were eluted from PGS pellet by boiling in the nonreducing sample buffer, separated by SDS-PAGE, and transferred to PVDF membranes (Millipore). Immunoblotting was performed using rabbit anti-K^b, rabbit anti-β2m, rabbit anti-TAP (Lybarger et al., 2003), and rabbit anti-HA (Covance), followed by horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Amersham Biosciences).

Generation of *CPXV203*-Deleted Virus

We followed a previously described protocol (Earl et al., 1998) with modifications. Genomic segments (500 bp long) flanking the *CPXV203* coding region were amplified and cloned into pBluscriptII KS (Stratagene). Between the flanking regions were inserted a cassette that expresses GFP under the VACV I1L promoter (TATTTAAAAG TTGTTTGGTGAACTTAA) (Liu et al., 2004) and two loxP sequences (ATAACTTCGTATAGCATACATTATACGAAGTTAT). The plasmid was transfected into CPXV-infected CV-1 cells using FuGENE6. *CPXV203*-deleted virus (CPXVΔ203) was purified from green fluorescent plaques by five rounds of plaque purification. For the deletion of the GFP cassette, CV-1 cells transiently expressing Cre recombinase were infected with CPXVΔ203, and the GFP-free CPXVΔ203 was purified from nonfluorescent plaques by five rounds of plaque purification. Deletion of CPXV203 was confirmed by western blot analysis using anti-CPXV203 antibody, which was raised in chicken by immunizing with C-terminal peptide CTNTKFDRSVYKTHMQKSKILHVKTEL (AVES) and HRP-conjugated goat anti-chicken IgY (AVES).

Supplemental Data

The Supplemental Data include four supplemental figures and can be found with this article online at http://www.cellhostandmicrobe.com/cgi/content/full/2/5/306/DC1/.

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